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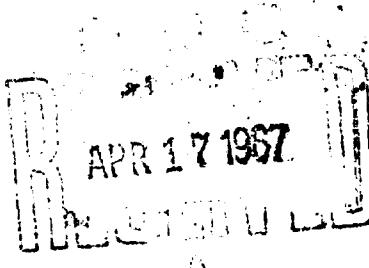
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TECHNICAL MANUSCRIPT 378

PROPERTIES OF VENEZUELAN
EQUINE ENCEPHALOMYELITIS VIRUS
GROWN IN VIVO

William T. Soper
Henry J. Hearn, Jr.

MARCH 1967



DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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PROPERTIES OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS
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Project 1C014501B71A

March 1967

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

ABSTRACT

One intracerebral passage of either the parent egg seed (PES) or an attenuated variant (10t) of the Trinidad strain of Venezuelan equine encephalomyelitis (VEE) virus in young adult mice produced progeny that were no longer differentiated unequivocally on the basis of plaque size. Plaques averaging about 2 mm in diameter, which was somewhat smaller than those formed by the PES virus and larger than those of the 10t strain, were formed by both strains. Seven serial passages of the PES virus in mouse brain failed to alter its virulence appreciably. In contrast, passage in mouse brain progressively changed the properties of the attenuated 10t strain. A substrain was isolated that possessed virulence similar to that of the PES virus and formed small plaques similar to those of the 10t strain. These findings showed a unique dissociation between the plaque size and virulence of the 10t strain. The new substrain differed from the PES virus and the 10t strain in its capacity for growth in mouse tissues following intraperitoneal inoculation. The substrain multiplied poorly in splenic tissue, which supports growth of the PES and 10t strains, but grew to high titers in the brain, which does not support appreciable growth of the 10t strain.

I. INTRODUCTION

Previous reports^{1,2} show that serial passage in vitro subjects Venezuelan equine encephalomyelitis (VEE) virus to environmental factors that favor the selection of progeny of significantly less virulence than that of the parent population. Moreover, tests on attenuated viral isolates show that the loss in virulence is accompanied by an inability of the virus to form large plaques under agar. Other experiments suggest, however, that passage in vivo may induce different effects. For example, despite the loss in virulence of our attenuated 9t strain for a number of laboratory animals, the hamster remains uniformly susceptible to lethal infection with this virus. Virulent large-plaque virus is readily isolated from the splenic tissue of moribund hamsters following injection of a high dilution of the small-plaque attenuated 9t strain.³ As another example, the St strain elicits few, if any, clinical symptoms when injected intraperitoneally in mice. Nevertheless, this virus is capable of growth in certain tissues, and small quantities of large-plaque virus are found among viral populations in the splenic tissue of inoculated mice.⁴

This report presents results of a further study of the changes in VEE virus populations after passage in vivo and a comparison of these changes with those that occur during passage in vitro. We introduce data showing that small-plaque formation and attenuation are not irreversibly associated after passage in vivo as previous studies in vitro indicate.

II. MATERIALS AND METHODS

A. VIRUS STRAINS

The parent egg seed (PES) of the Trinidad strain of VEE virus,⁵ prepared after 13 passages in embryonated eggs, was used in these studies. The attenuated strain was isolated from L cell monolayer culture 8 months after infection with the PES strain. The isolate was serially passed ten times in fresh L cells and designated the 10t strain; it differs from the 9t strain⁶ in that it received one additional passage in L cells.

B. IN VITRO CULTURE METHODS

Monolayers of chick fibroblast cells were used for plaque assay and passage of selected strains. The techniques have been described elsewhere.¹

C. PREPARATION OF INFECTED TISSUES

Mice were sacrificed and the brain and spleen were removed aseptically. The tissues were ground in a Ten Broeck grinder and diluted with sterile Bacto heart infusion broth to make 10% suspensions. Samples were stored in glass ampoules in a dry ice chest until assays were performed.

D. VIRUS ASSAY

Viral strains and infected tissue preparations were titrated by the intracerebral (IC) and intraperitoneal (IP) routes in 12- to 14-g Swiss white mice. Titer values were calculated by the Reed-Muench method⁷ and expressed as the MICLD₅₀ per ml and MIPLD₅₀ per ml, respectively. The degree of virulence, which has been useful in characterizing VEE viral strains,² was determined from these basic titrations. Because attenuation was marked by an inability to produce lethality by the IP route, the virulence level of a virus preparation was expressed by the log₁₀ difference between the MICLD₅₀ titer and the MIPLD₅₀ titer (MICLD₅₀ - MIPLD₅₀). For example, the virulent PES strain showed a value for MICLD₅₀ - MIPLD₅₀ of 2 logs or less; this difference was greater than 7.0 logs for the IP-attenuated 10t virus. Between these two extremes, the difference between the titers increased with loss of virulence or decreased as virulence was restored.

III. RESULTS

A. PASSAGE OF PES AND 10t STRAINS IN MICE

The effect of IC passage in mice on the properties of the PES and 10t viral strains is shown in Table 1. With the virulent PES strain, titers of 10^{10.5} MICLD₅₀ and 10^{8.1} MIPLD₅₀ per ml were obtained; the virulence level of the population was 1.4 logs. During seven serial IC passages of the PES strain in mice, the virulence of mouse brain populations remained approximately the same (1.1 to 1.6 logs). It was evident, however, that passage of the PES strain in mice resulted in progeny that formed somewhat smaller plaques than did the PES strain. As examples, the first- and seventh-passage mouse brain preparations, designated PES-mb and PES-7mb, respectively, are shown in Table 1.

TABLE 1. LETHALITY OF VIRULENT PES AND ATTENUATED 10t STRAINS OF VEE VIRUS AFTER PASSAGE IN MICE^{a/}

Virus Strain	Virus Titers, log ₁₀		Virulence Level, log ₁₀	Plaque Diameter, mm
	MICLD ₅₀ ^{b/}	MIPLD ₅₀ ^{c/}		
PES	10.5	9.1	1.4	3-6
PES-mb	10.3	9.1	1.2	2-3
PES-7mb	10.1	8.7	1.4	2-3
10t	7.6	<0.6 ^{e/}	>7.0	0.5-1.5
10t-mb	7.4	2.4	5.0	1-3

a. Intracerebral passage in 12- to 14-g mice.

b. Titrated intracerebrally in 12- to 14-g mice.

c. Titrated intraperitoneally in 12- to 14-g mice.

d. Log₁₀ difference between MICLD₅₀ and MIPLD₅₀ titers.

e. No lethality at dilutions of 10⁰ through 10⁻⁷.

In contrast, the attenuated 10t strain titered 10^{7.8} MICLD₅₀ per ml in mice but was not lethal by the IP route (Table 1); the MICLD₅₀ - MIPLD₅₀ value was high (>7.0 logs) and the virus formed small plaques (0.5 to 1.5 mm diameter). One passage of this strain in mouse brain (10t-mb), however, caused a slight elevation in IP virulence; the MICLD₅₀ - MIPLD₅₀ decreased from >7.0 logs to 5.0 logs. Plaques increased to a range of 1 to 3 mm; the majority were 2 mm in diameter.

Thus, passage of the PES strain in mice yielded viral populations that showed no appreciable change in virulence but did form plaques of slightly reduced size. The 10t-mb population showed an increase in IP lethality and a slight increase in plaque size. Contrary to the obvious differences in size between plaques of the unpassed PES and 10t strains, morphological differences between plaques of these strains were comparatively indistinct after the first passage in mouse brain.

B. DERIVATION OF A VIRAL POPULATION WITH DISSOCIATED PLAQUE SIZE AND VIRULENCE PROPERTIES

Because one passage of the 10t strain in mouse brain increased the plaque size and virulence of the progeny, studies were continued to determine whether additional passages influenced these properties. These passages and the subsequent changes in virulence are shown graphically in Figure 1; detailed results of mouse and plaque assays appear in Table 2.

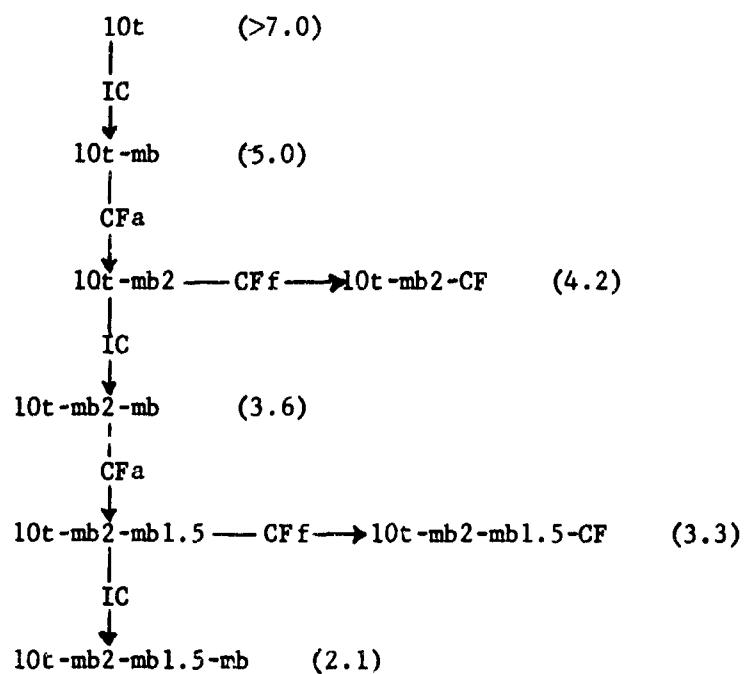


Figure 1. Derivation of a New VEE Virus Substrain.
Passage of indicated virus preparation in chick fibroblasts under agar (CFA) for plaque isolation; in chick fibroblast fluid culture (CFf); or intracerebrally (IC) in mice. The virulence level (MICLD₅₀ - MIPLD₅₀) at each passage is shown in parentheses.

TABLE 2. TITERS OF VIRUS DERIVED FROM THE ATTEN. TIED 10t STRAIN
AFTER SERIAL PASSAGE IN VARIOUS HOST SYSTEMS

Virus Strain	Virus Titers, \log_{10}		Virulence Level, \log_{10}	Plaque Diameter, mm
	MICLD ₅₀ ^{a/}	MIPLD ₅₀ ^{b/}		
10t-mb ^{d/}	7.4	2.4	5.0	1-3
10t-mb2-CF ^{e/}	7.1	2.9	4.2	1-2
10t-mb2-mb ^{f/}	7.9	4.3	3.6	1-2
10t-mb2-mb1.5-CF ^{g/}	7.7	4.4	3.3	1-2
10t-mb2-mb1.5-mb ^{h,i/}	8.7	6.6 ^{j/}	2.1	1-1.5

- a. Titrated intracerebrally in 12- to 14-g mice.
- b. Titrated intraperitoneally in 12- to 14-g mice.
- c. \log_{10} difference between MICLD₅₀ and MIPLD₅₀ titers.
- d. 10t strain passed intracerebrally in 12- to 14-g mice.
- e. One plaque passage of 10t-mb virus in chick fibroblasts; virus isolated from a 2-mm plaque and passed in chick fibroblast (CF) fluid culture.
- f. Same plaque virus as in (e), except that it was passed in mouse brain instead of in CF fluid culture.
- g. One plaque passage of 10t-mb2-mb virus in chick fibroblasts; virus isolated from a 1.5-mm plaque and passed in CF fluid culture.
- h. Same plaque virus as in (g), except that it was passed in mouse brain instead of in CF fluid culture.
- i. Brain and splenic tissue were removed from mice during the IC and IP titrations of this virus strain. Data obtained with these tissues are shown in Table 3.
- j. Interference: lowest dilutions used in the titration failed to be lethal for all of the injected mice.

When virus from a 2-mm plaque of the 10t-mb preparation was passed IC in mice, the resultant virus (10t-mb2-mb) showed an increase in virulence as the MICLD₅₀ - MIPLD₅₀ value was reduced from 5 logs, shown by the 10t-mb population, to 3.6 logs. Interestingly, passage of the same plaque virus in chick fibroblast fluid culture (10t-mb²-CF) did not produce the same marked changes; the virulence level was 4.2 logs. Plaques formed by the 10t-mb2-mb virus ranged from 1 to 2 mm in diameter with the majority 1.5 mm. Studies were continued with 1.5-mm plaque virus (10t-mb2-mb1.5).

IC passage of the 1.5-mm plaque virus in mice resulted in infected mouse brain (10t-mb2-mb1.5-mb) with a titer of $10^{8.7}$ MICLD₅₀ per ml and $10^{8.8}$ MIPLD₅₀ per ml (Table 2). The virulence level (2.1 logs) of this preparation approached the value shown by the PES strain. In contrast, parallel passage of the 1.5-mm plaque virus in CF fluid culture (10t-mb2-mb1.5-CF) yielded progeny whose level of virulence (3.3 logs) was not significantly different from that of the parent virus (10t-mb2-mb), for which the MICLD₅₀ - MIPLD₅₀ was 3.6 logs.

In spite of the similarity in virulence shown by the 10t-mb2-mb1.5-mb and the unmodified PES virus, differences still were apparent. One of these was revealed by the fact that the 10t-mb2-mb1.5-mb virus displayed marked interference upon IP injection in mice at low dilutions (Table 2), a phenomenon not previously observed in our laboratory with VEE virus. As a result, the MIPLD₅₀ titer of the 10t-mb2-mb1.5-mb virus, calculated by the Reed-Muench method, appears approximately 1 log lower in Table 2 than it would have been in the absence of the interference. Considering a second difference, the property of plaque size, the 10t-mb2-mb1.5-mb sub-strain formed small plaques despite its increased virulence, indicating that the 10t strain had undergone a unique dissociation of plaque size and virulence. Thus, the 10t-mb2-mb1.5-mb strain represented a new VEE virus substrain that was different from any other we have isolated.

C. CHARACTERIZATION OF THE 10t-mb2-mb1.5-mb SUBSTRAIN

In other experiments performed in our laboratory, the capacity for growth in mouse tissues in vivo has been useful in the characterization of viral substrains. The results of studies on the distribution and properties of virus in mouse tissues after IC and IP injection of the 10t-mb2-mb1.5-mb strain are shown in Table 3; these tissues were obtained from mice used in the titrations of 10t-mb2-mb1.5-mb shown in Table 2.

After IC or IP injection of relatively low doses of the 10t-mb2-mb1.5-mb virus, brain tissue contained high concentrations of virus; the splenic tissue contained no demonstrable virus. Virus from the brain tissue was highly virulent, like the PES strain, but it formed plaques typical of the unmodified 10t virus. No interfering effect, such as that shown in Table 2, was exhibited when this virus was titrated IP in mice. When tissues were examined from mice injected IC or IP with 3- to 4-log higher doses of the 10t-mb2-mb1.5-mb virus, the brain tissues contained high viral titers that were comparable to those obtained when limiting dilutions were used. In contrast to the results with the low dose, some virus appeared in splenic tissue and the interference effect was observed in the IP titrations of both brain and splenic tissue.

TABLE 3. GROWTH OF THE 10t-mb2-mb1.5-mb VIRUS STRAIN
IN MOUSE BRAIN AND SPLEEN^{a/}

Dilution and Route of Injection ^{b/}	Tissue ^{c/}	Virus Titers, log ₁₀		Virulence Level, log ₁₀ ^{d/}	Plaque Diameter, mm
		MICLD ₅₀ ^{d/}	MIPLD ₅₀ ^{e/}		
10 ⁻⁷ IC	day 7	Brain	8.0	6.1	1.9
		Spleen	-g/	-	-
10 ⁻⁶ IP	day 7	Brain	7.0	6.0	1.0
		Spleen	-	-	-
10 ⁻³ IC	day 3	Brain	9.0	7.0 ^{h/}	2.0
		Spleen	4.1	1.6 ^{h/}	2.5
10 ⁻³ IP	day 4	Brain	8.0	6.0 ^{h/}	2.0
		Spleen	3.7	1.9 ^{h/}	1.8

- a. Tissues obtained from mice during the IC and IP titrations of 10t-mb2-mb1.5-mb (Table 2).
- b. Titer of 10t-mb2-mb1.5-mb substrain was 10^{8.7} MICLD₅₀ per ml.
- c. Tissues harvested at indicated day postinjection.
- d. Titrated intracerebrally in 12- to 14-g mice.
- e. Titrated intraperitoneally in 12- to 14-g mice.
- f. Log₁₀ difference between MICLD₅₀ and MIPLD₅₀ titers.
- g. No virus detected.
- h. Interference: lowest dilutions used in the titration failed to be lethal for all of the injected mice.

The 10t-mb2-mb1.5-mb substrain could be differentiated both from PES virus and the 10t parent strain on the basis of its neurotropism following extraneural inoculation (Table 4). After the IP injection of PES virus in mice, 10^{8.7} MICLD₅₀ per ml and 10^{6.0} MICLD₅₀ per ml were obtained, respectively, in the brain and splenic tissues of moribund mice. Upon IP injection of the 10t strain, maximal viral yields of 10^{2.8} and 10^{7.0} MICLD₅₀ per ml were found in brain and spleen. In contrast, the 10t-mb2-mb1.5-mb virus reached 10^{7.0} MICLD₅₀ per ml in the brain, but no virus could be detected in spleen homogenates tested either in cell cultures or in mice. The variant, unlike the PES or 10t viruses, showed a reduced affinity for splenic tissue; its degree of neurotropism, however, approached that of the PES virus.

TABLE 4. COMPARATIVE GROWTH OF THREE STRAINS OF VEE VIRUS
IN MOUSE BRAIN AND SPLEEN AFTER INJECTION IP

Virus Strain	Strain Lethality ^a /	Virus Titer, MICLD ₅₀ (log ₁₀)	
		Brain	Spleen
PES ^b /	+	8.7	6.0
10t ^c /	-	2.8	7.0
10t-mb2-mb1.5-mb ^d /	+	7.0	neg ^e /

- a. Lethal (+) or nonlethal (-) for mice by the IP route.
- b. 12- to 14-g mice injected IP with approximately 80 MICLD₅₀ of the virulent PES strain; tissues harvested when mice became moribund.
- c. 12- to 14-g mice injected IP with approximately 80 MICLD₅₀ of the attenuated strain; tissues harvested at various intervals postinjection; titers represent highest values obtained.
- d. 12- to 14-g mice injected IP with approximately 1,100 MICLD₅₀ of the substrain; tissues harvested when mice became moribund.
- e. No virus detected upon inoculation of splenic homogenate either in cell culture or in mice.

IV. DISCUSSION

Previous studies¹ with VEE virus showed that passage of the virulent PES strain *in vitro* selects virus possessing a reduced ability both to cause lethal infections in mice and to form large plaques. This phenomenon has been observed by other investigators working with several arboviruses; recent accounts review these findings.^{2,3} Our present data reveal a marked contrast between the properties of VEE virus after passage *in vivo* and those found after passage in cell cultures.

During seven serial IC passages in young adult mice, the high virulence of the PES virus is unchanged; the mouse brain virus does form smaller plaques (2 to 3 mm in diameter) than those of the unmodified PES strain (3 to 6 mm). These findings agree with those of Heydrick et al.,² who find no loss in virulence, but do report a decrease in plaque size after ten serial passages of PES virus in suckling mouse brain.

On the other hand, we find that a single mouse brain passage of the attenuated 10t virus is sufficient to increase the virulence of the strain. Further passage, alternating between plaque isolation in chick fibroblasts and passage in mouse brain, continues to induce a stepwise increase in virulence but retains the 10t strain small-plaque characteristic. A new substrain, 10t-mb2-mb1.5-mb, ultimately emerges that combines the small-plaque property of the unmodified 10t virus with the characteristic virulence of the PES virus.

The trend of these stepwise changes can be interrupted when plaque-isolated virus is passed in chick fibroblast fluid cultures instead of in mouse brain. Comparative studies show that growth in these cultures fails to increase virulence appreciably above its level at the previous mouse brain passage. In Figure 1 compare the CF and mb passages of 10t-mb2 with its parent, 10t-mb. These observations are consistent with results showing that growth of VEE virus in chick fibroblast fluid cultures is selective for virus possessing avirulent properties.¹

On this basis, the alternate use of growth *in vitro* for plaque isolation coupled with passage in mouse brain may have influenced our results. The *in vitro* growth may be responsible for retaining small plaque size; other 10t virus properties change in response to growth *in vivo*. In this respect, it is interesting that the PES and 10t populations exhibit overlapping plaque morphology after one mouse brain passage. However, further serial passages of PES in mouse brain without plaque passage continue to produce virulent virus forming 2- to 3-mm plaques. The question arises whether the alternate use of plaque passage and passage in mice of PES virus would yield small-plaque virus with properties completely indistinguishable from those of the neurotropic 10t-mb2-mb1.5-mb virus substrain.

One important difference between the 10t-mb2-mb1.5-mb substrain and the PES strain is that the former grows poorly in splenic tissue despite its lethality for mice IP. It is interesting that both the PES virus and the 10t strain, from which the substrain is derived, show comparable growth in the spleen. Despite its activity in the spleen, the attenuated 10t strain does not multiply appreciably in the brain after extraneural injection; thus, IP attenuation of this strain is probably related to its inability to proliferate to an appreciable extent in brain tissue. On the other hand, the loss of viscerotropism by the 10t virus during mouse brain passage is accompanied by restoration of neurotropism, which undoubtedly accounts for the lethality of the 10t-mb2-mb1.5-mb virus injected IP.

A second difference between the PES virus and the 10t-mb2-mb1.5-mb substrain is expressed by the apparent interference that occurs when the latter is injected at a high virus dose. In our experience, during many tests with the PES strain of VEE virus, we have never observed this phenomenon. Previously, it was suggested that interference phenomena might have some influence on the avirulence of attenuated VEE virus.⁴ It also has been postulated that interference might be involved in the resistance of mice to lethal doses of VEE, Eastern equine encephalomyelitis, and vaccinia viruses shortly after immunization with attenuated VEE virus.⁵

The decreased lethality that is immediately evident after IP injection of a high dose of the 10t-mb2-mb1.5-mb virus may be another manifestation of interference phenomena; the requirement for a high dose indicates that the interfering particles are present in the viral population at very low concentration. The expression of interference also may be related to the route of inoculation; injection of the high dose by the IC route does not produce decreased lethality. Interfering virus is subsequently present, however, in the brain and splenic tissue of these mice and is demonstrable on IP injection in mice at a high dose (Table 3). If interfering virus exists, it is not definitely known at the present time to what extent this virus may proliferate in vivo and in which tissues growth is optimal.

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